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Note

Mobility of various buffers in reversed-phase thin-layer chromatography

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The application of buffers in planar chromatography is as old as paper chromatography (PC) and thin-layer chromatography (TLC) themselves. Buffered systems were used to chromatograph dissociable compounds (salts of weak acids or bases) under definite acid–base conditions[1], and were applied either to impregnate the paper[2] or the thin-layer support[3,4] or as a component of the mobile phase.

In the impregnation technique it was accepted that the buffering effect is evenly distributed over the whole plate surface and the latter was suposed to show the same pH value as that of the buffer. The effect of the pH of the buffer on the support (silica, cellulose) was not taken into account. To prevent a change in buffer composition by elution of its components during the flow of the mobile phase, most authors saturated the latter with the buffer solution prior to development.

The use of buffer solutions as mobile phases in PC can be demonstrated by salting-out chromatography[5] and by pH chromatography[6,7]. Buffered mobile phases have frequently been applied in TLC and in reversed-phase (RP)TLC systems for analytical separation[8] or for lipophilicity determination[9–11]. The application of buffered mobile phases is also very common in ion-exchange TLC[12–15].

It is well known both in PC and TLC that the composition of multi-component mobile phases is changed during their flow through the suport. This is the case not only with solvent mixtures[16–19] but also with salt solutions[5]. Thus Hagdahl and Tiselius[5] using 1 *M* disodium hydrogenphosphate and 2 *M* sodium dihydrogenphosphate as mobile phases in so-called salting-out PC stated that a concentration gradient with the maximum at the front should be established on the chromatogram. Therefore, during the flow of the mobile phase through the support, its composition and consequently its pH value is expected to change. The type of support and its acid–base properties should, however, also be taken into consideration. These phenomena usually do not decrease the performance of the chromatographic system, on the contrary, the established pH gradient can work positively and improve the separation.

In quantitative structure–activity relationship studies the use of buffer solutions as mobile phases in both normal-phase and RP systems is a problem in that

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the expected pH change of the support due to the mobile phase decomposition and the effect of the acid–base properties of the support could lead to false results. Here, buffer solutions are used as mobile phases on untreated[11] layers or on layers impregnated with apolar stationary phases (liquid paraffin, silicone oil, etc.)[9,10] and the R_M values determined are used as the basis of calculations to correlate the lipophilicity of analytes with their biological activities and to design new bioactive compounds.

Recent research indicates that the retention of analytes is also influenced by the quality of the buffer cations[20], and the actual pH values depend considerably on the quantity of organic modifier in the eluent[21]. It was found[22] that by using a veronal buffer of pH 8.8 as mobile phase on silica layers the pH was 8 at the starting line and decreased successively to pH 4 at the solvent front, at a distance of 10 cm from the start. The pH value of undeveloped silica was also 4. This difference on cellulose layers under identical conditions was only 1 pH unit.

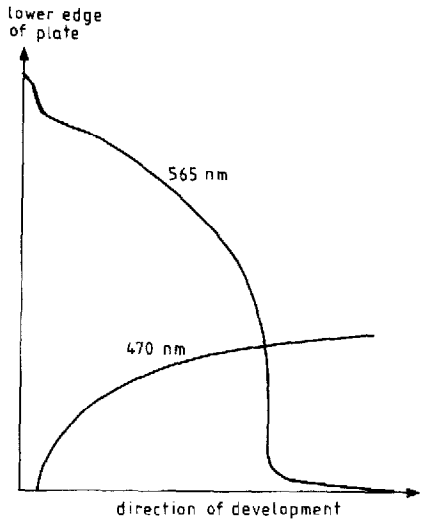
The objectives of our work were to study in more detail the retention of various buffering ions by the layer and to determine their buffering capacity.

EXPERIMENTAL

Polygram Sil G plates (Macherey-Nagel, F.R.G.) were impregnated with paraffin oil as described[23]. The plates were developed with water and water–methanol mixtures (4:1, 3:2, 2:3 and 1:4, v/v) containing final buffer conentrations of 0.1, 0.2, 0.3 and 0.4 *M*. The pH value of the buffers (sodium phosphate, sodium acetate and sodium diethylbarbiturate) was set to 8.50. After development the movement of the alkalinity front was detected by spraying half the plate with a 0.1% solution of phenol red indicator (red at pH 8.50 and yellow at the pH value of the impregnated silica). The evaluation was carried out by a Shimadzu dual-wavelength TLC scanner CS-930 at 565 (red) and 470 nm (yellow). In case of veronal buffers the movement of diethylbarbituric acid was detected on the other half of the plate at 250 nm. Phosphate ions were detected by use of the ammonium molybdate–tin(II) chloride reagent[24] and then evaluated at 600 nm.

As the mobility of the alkalinity front generated by sodium acetate was high it was unnecessary to detect the acetate ions separately. Each experiment was performed in quadruplicate.

We assumed that the hypothetical spots were placed on the plates at 15 mm above the eluent level and calculated the R_F value of the alkalinity front accordingly. As these R_F values indicated that not only the water–methanol ratio but also the final concentration of buffer influences the mobility and the exact type of correlation (linear or logarithmic) between independent (methanol and buffer concentration) and dependent variables (R_F value of alkalinity front) was not previously established we used stepwise regression analysis to elucidate this problem[25]. The R_F values were taken as dependent, the linear and logarithmic forms of methanol (%) and buffer concentrations (M) as independent variables. The number of accepted variables was not limited, the partial F value of the variables being set to $F = 1$. The calculations were carried out for sodium acetate and sodium phosphate separately. The low mobility of veronal buffers makes these data irrelevant from a practical point of view; therefore they were omitted from the calculations.



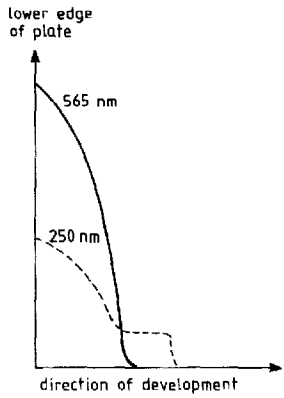


Fig. 1. Movement of the alkalinity front detected at two wavelengths. R_F of alkalinity front: 0.50. Final concentration of phosphate buffer: 0.4 *M*. Eluent: water.

Fig. 2. Movement of veronal buffer detected at two wavelengths. R_F of alkalinity front: 0.12. Final concentration of veronal buffer: 0.4 *M*. Eluent: water.

As it was previously reported that the extent of impregnation exerts a considerable effect on the retention behaviour of RPTLC layers[26], Polygram plates impregnated with 1, 2.5, 5 and 10% paraffin oil were developed in 0.4 *M* buffer solutions in water as eluent and then evaluated as described above. Unimpregnated plates served as controls.

RESULTS AND DISCUSSION

A typical chromatogram evaluated at 565 and 470 nm is shown in Fig. 1. As the alkaline form of phenol red also shows a marked absorption at 470 nm the change in pH of the plate surface cannot be adequately followed at this wavelength. Consequently the determination of the alkalinity front was carried out at 565 nm. The lower edge of the plates indicates in each case the first point of the plate above the eluent level. Veronal buffers exhibited poor mobilities in each eluent (Fig. 2), however, the diethylbarbituric acid moved further on the plates than the alkalinity. This somewhat surprising observation can be explained by the assumption that free silanol groups exhibit higher affinity to sodium ion than to diethylbarbituric acid. The adsorption of sodium ions makes the silica surface alkaline and the veronal buffer depleted of sodium ions moves alone. The R_F value of the alkalinity front decreased with increasing methanol concentration. This is caused by the fact that the decreasing dielectric constant of the eluent (higher methanol content) suppresses the dissociation of veronal. The undissociated form is more lipophilic and is therefore more strongly retained by the reversed phase.

The separation of the anionic and cationic parts of the buffer was also observed in sodium phosphate buffers (Fig. 3) and can be explained as in the case of veronal buffers. We have, however, to take into consideration that sodium ions can be re-

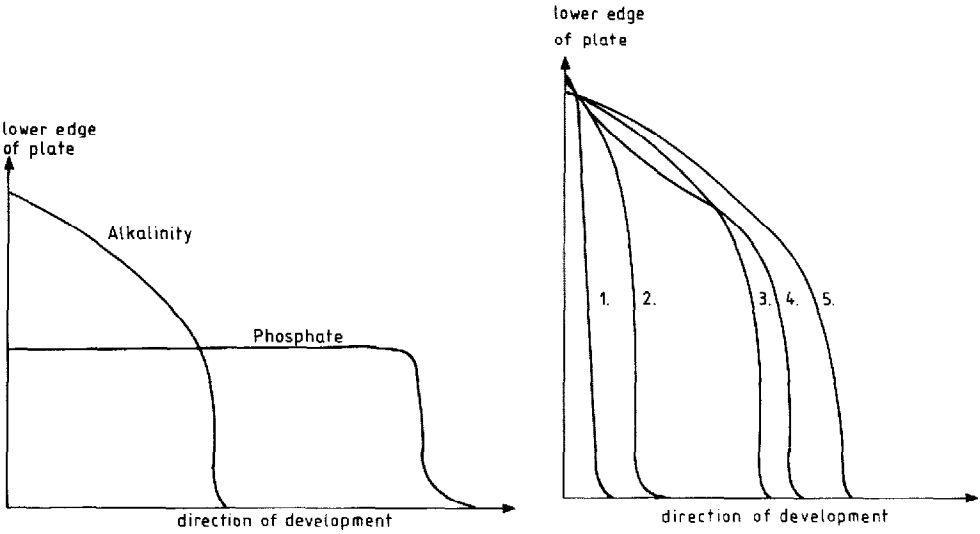


Fig. 3. Movement of alkalinity and phosphate fronts. R_F of alkalinity and phosphate front: 0.49 and 0.92 respectively. Final concentration of phosphate buffer: 0.3 *M*. Eluent: water.

Fig. 4. Effect of the eluent composition on the position of the alkalinity front. Final concentration of phosphate buffer: 0.3 *M*. Eluent: 1, water–methanol (1:4); 2, water–methanol (2:3); 3, water–methanol (3:2); 4, water–methanol (4:1); 5, water.

tained not only by the free silanol groups uncovered by paraffin oil but also by the paraffin oil itself resulting in the same dissociation. Our other investigations showed that, under similar RPTLC conditions, monovalent cations exhibit high apparent "lipophilicity". The position of the alkalinity front depends also in this case on the methanol content, decreasing with increasing methanol concentration (Fig. 4). The distribution of pH is uneven; it decreases very slowly near to the lower edge of the plate, but ends with a well defined, abrupt change. Sodium acetate buffers move readily with the eluent, and are hardly influenced by the methanol concentration of

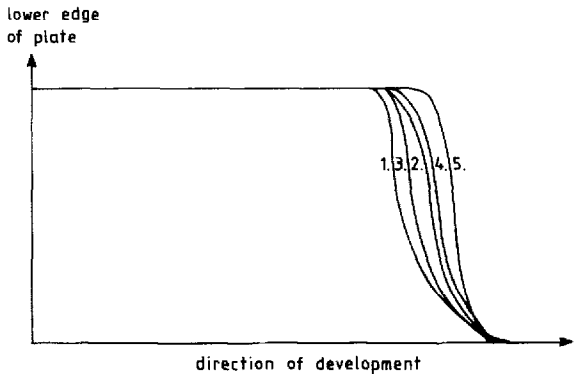


Fig. 5. Effect of the eluent composition on the position of the alkalinity front. Final concentration of acetate buffer: 0.3 *M*. Eluents as in Fig. 4.

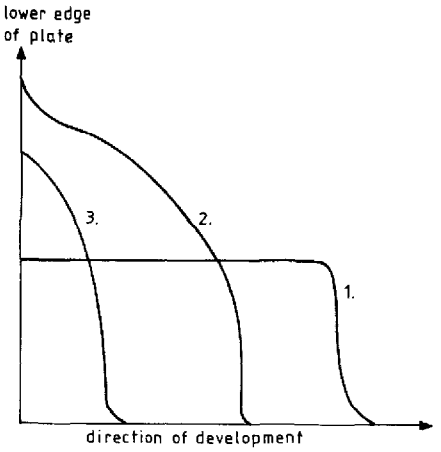


Fig. 6. Positions of the alkalinity fronts generated by various buffers. Final concentration of buffers: 0.4 *M*. Eluent: water. Buffers: 1, acetate; 2, phosphate; 3, veronal.

the eluent (Fig. 5). The distribution of pH is even; the change is very abrupt and it is near to the eluent front.

The behaviour of the three buffers is compared in Fig. 6. The low mobility of veronal buffers (highest R_F value observed: 0.12) makes them unsuitable to buffer similar RPTLC systems. Phosphate has an higher mobility (up to $R_F = 0.50$), how-

TABLE I

DEPENDENCE OF THE POSITION OF THE ALKALINITY FRONT IN PHOSPHATE, y_1, AND ACETATE, y_2, BUFFERS ON THE METHANOL, x_1, AND BUFFER, x_2, CONCENTRATION OF THE ELUENT

Results of stepwise regression analysis.

$y_1 = a + b_1x_1 + b_2x_2$ (A)

$y_2 = a + b_1x_1 + b_2 \log x_2$ (B)

*Parameter**	*Eqn. A*	*Eqn. B*
a	0.31	0.83
b_1	$-5.10 \cdot 10^{-3}$	$-1.27 \cdot 10^{-3}$
b_2	0.42	$5.21 \cdot 10^{-2}$
r^2	0.8753	0.7386
b'_1 (%)	75.53	75.37
b'_2 (%)	24.47	24.63
s	$6.71 \cdot 10^{-2}$	$2.45 \cdot 10^{-2}$
s_{b1}	$5.30 \cdot 10^{-4}$	$1.91 \cdot 10^{-4}$
s_{b2}	0.13	$2.42 \cdot 10^{-2}$
F	51.07	24.01
t_1	9.61	6.59
t_2	3.11	2.15

$n = 20$, $F_{99.9\%} = 10.66$, $t_{95\%} = 2.11$, $t_{99\%} = 2.90$, $t_{99.9\%} = 3.97$

* b'_1 and b'_2 = Contribution of independent variables x_1 and x_2 to the position of the alkalinity front (so-called path coefficients); s, s_{b_1} and s_{b_2} = standard deviations of y_1, x_1 and x_2; t_1 and t_2 = indicators of the significance level of b_1 and b_2.

ever, its mobility depends heavily on the organic phase concentration. We suggest that before applying phosphate buffers it is advisable to check that the compounds to be separated are in the buffered zone. Our data clearly show that acetate buffers are the most suitable in RPTLC to produce a buffering effect which is evenly distributed along the plates. The results of stepwise regression analysis are compiled in Table I. The calculations entirely support our qualitative conclusions based on the chromatograms.

In both cases the independent variables (methanol and buffer concentrations had a significant) influence (significance level over 99.9%) on the R_F value of the alkalinity front. The r^2 values show that the change in the independent variables is responsible for about 80% of the change in R_F value. The R_F value decreased with increasing methanol concentration (negative b_1 values) and increased with increasing buffer concentration (positive b_2 values), however, these effects were higher in the case of phosphate buffer (b_1 and b_2 values of eqn. A are higher than the corresponding values of eqn. B). The difference in the intercept value (position of alkalinity front at zero methanol and buffer concentrations), a, shows that acetate buffer ($a = 0.81$) has a higher mobility than the phosphate one ($a = 0.31$). The significant t values indicate that both variables have a significant effect on the R_F value of the alkalinity front, however, the impact of the methanol concentration is about three times higher than that of the buffer concentration (see path coefficients b_1' and b_2'). The movement of phosphate ions depended only on the methanol concentration:

$$R_{F\,\text{phosphate}} = 0.92 - (3.69 \pm 0.30) \cdot 10^{-3} \cdot \text{methanol concentration}$$
$$r_{\text{calc.}} = 0.9458 \qquad r_{99.9\%} = 0.6787$$

Summarizing our results, we established that the buffering capacity of veronal, phosphate and acetate buffers is different in RPTLC. The movement of the alkalinity front depends strongly on the organic phase ratio and to a lesser extent on the buffer concentration of the eluent. The buffering capacity increased in the order veronal < phosphate < acetate. Our results concerning the effect of the extent of impregnation on the retention of buffer ions are compiled in Table II. The data show that the retention increases in each case with increasing extent of impregnation. This finding is in good accordance with the fact that the charge in the ratio of the parti-

TABLE II

R_F VALUES OF BUFFER ION FRONTS ON RPTLC PLATES IMPREGNATED TO DIFFERENT EXTENTS

	Paraffin oil concentration (%)				
	0	*1*	*2.5*	*5*	*10*
Phosphate anion	1.00	0.98	0.99	0.96	0.94
Alkalinity caused by phosphate	0.76	0.73	0.71	0.64	0.61
Veronal anion	1.00	0.58	0.37	0.29	0.20
Alkalinity caused by veronal	0.12	0.10	0.07	0.07	0.05
Alkalinity caused by acetate	0.90	0.89	0.85	0.85	0.82

tioning phases (the mobile water phase is identical, the quantity of adsorbed paraffin oil increases) modifies the partition in favour of the phase present in higher quantity. However, this effect is fairly low and it does not change the capacity order of the buffers established above.

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